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14. ABSTRACT

The **purpose** of this project is to investigate whether epigenetic mechanisms may contribute to reduced expression of the tumor suppressor gene BRCA-1 in sporadic breast cancers. The **scope** is to test the role of xenobiotics and food compounds that bind the aromatic hydrocarbon receptor (AhR). AhR-ligands include the dioxin-like and tumor promoter 2,3,7,8 tetrachlorobenzo-p-dioxin (TCDD). The activated AhR regulates transcription through binding to xenobiotic response elements (XRE=GCGTG) and interactions with transcription cofactors. **Major findings**: We report that 1) in pilot experiments, the in vitro treatment of estrogen receptor (ER)-positive and BRCA-1 wild-type breast epithelial cells with TCDD attenuates 17β-estradiol (E2)-dependent stimulation of BRCA-1 protein and induces hypermethylation of a CpG island spanning the BRCA-1 transcriptional start site of exon-1a; 2) TCDD enhances the association of the AhR, DNA methyl transferases (DNMT)1, DNMT3a, and DNMT3b; methyl binding protein (MBD)2; and tri-methylated H3K9 (3meH3K9) with the BRCA-1 promoter; 3) The phytoalexin resveratrol, selected as a prototype dietary AhR antagonist, antagonizes at physiologically relevant doses (1 μmol/L) the TCDD-induced repression of BRCA-1 protein, BRCA-1 promoter methylation, and the recruitment of the AhR, MBD2, 3meH3K9, and DNMTs (1, 3a, and 3b); 4) In-utero treatment with TCDD increases the percentage of terminal end-buds in female offspring; 5) We have optimized measurements of BRCA-1 expression in mammary tissue. Measurements are in progress to examine the effects in utero of TCDD exposure on BRCA-1 expression and promoter hypermethylation in mammary tissue of female offspring. **Significance**: Taken together, these observations provide preliminary evidence AhR activation is a risk factor in the etiology of sporadic breast cancer through BRCA-1 silencing and provide the molecular basis for prevention programs based on dietary AhR antagonists.

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Introduction

Subject: Epigenetic alterations such as histone and DNA modifications have been proposed to play a key role in the onset of a variety of tumors by inducing stable, heritable changes in gene expression [1-3]. **Purpose**: Test if ligands of the aromatic hydrocarbon receptor (AhR), including dioxins contribute to sporadic breast cancer. The **scope** is to test whether activation of the AhR leads to specific histone and DNA modifications that cause silencing of the BRCA-1 gene and increase mammary cancer risk. Also, this project investigates whether these modifications can be reversed with dietary antagonists of the AhR. **Hypothesis:** In utero exposure to AhR ligands leads to chromatin modifications that silence the BRCA-1 gene in female offspring increasing the susceptibility to breast cancer, whereas these epigenetic alterations might be reversed through supplementation with dietary antagonists of the AhR. The **rationale** for this proposal stems from evidence the dioxin compound and AhR ligand TCDD induces the recruitment of the activated AhR to the BRCA-1 promoter and this effect is paralleled by (i) reduced occupancy at the BRCA-1 gene of histone acetyltransferases (HATs); (ii) increased recruitment of histone deacetylases (HDACs); and (iii) repression of BRCA-1 promoter activity [4]. The removal of acetyl groups from histones along with other histone modifications may result in downstream changes that culminate with DNA methylation, the formation of heterochromatin, and heritable silencing of the BRCA-1 gene; (iv) in utero exposure to the AhR ligand and dioxin TCDD altered normal mammary gland differentiation and increased the susceptibility to mammary carcinogenesis in female offsprings [5]. Therefore, administration of selected dietary modulators of the AhR during the prenatal period may exert protective effects against prenatal AhR-dependent epigenetic silencing of BRCA-1. **Specific Aims** are designed to investigate in a rat mammary tumor model whether in-utero exposure to the AhR agonist TCDD silences BRCA-1 expression in adult offspring thus increasing the susceptibility to mammary carcinogenesis, and the reversal effects of the dietary AhR antagonist, resveratrol (RES). **Study Design**. **Objective 1:** Timed pregnant rat females were assigned to four groups: Group 1, gavaged with 1 μg TCDD/kg body weight on day 15 post-conception; Control Group 2, gavaged with an equivalent volume of sesame oil (~0.2 ml/animal); Group 3, prophylactic group receiving RES from 7 days post breeding and then continued until the end of gestation; and Group 4 receiving RES at 7 days before gavage with TCDD and then continued until the end of gestation. Measurements include changes in expression of BRCA-1, BRCA-1 promoter methylation and chromatin remodeling including histone modifications and chromatin accessibility. **Objective 2**: six-weeks old female offsprings born from control mothers or mothers exposed to TCDD, RES or TCDD plus RES as described in Objective 1 will be administered a single dose of DMBA (10mg/rat administered by gavage in corn oil). Measurements will include tumor data (incidence, multiplicity, latency, volume), histopathology, BRCA-1 promoter methylation, histone modifications, and chromatin remodeling.

Body

This Annual progress report refers to activities of *Task 1* of the proposal, which were planned for the first 12 months of the project.

Objective a) of The Statement of Work for this task includes measurements of BRCA-1 expression by RT-PCR and western blotting. To accomplish this objective we have developed preliminary experiments that tested: 1) the ability of estrogen to induce BRCA-1; 2) the ability of TCDD to antagonize estrogen induction; and 3) the effects of the antagonist resveratrol against the repressive effects of TCDD. These measurements established a working platform for the subsequent experiments and validated the technical procedures.

To examine the dose-response effects of TCDD on E2-regulated BRCA-1 protein, we pretreated MCF-7 cells with various doses of TCDD followed by cotreatment with E2. Western-blot analysis of cell lysates indicated a U-shaped relationship with increased BRCA-1 expression at lower (< 5 nmol/L) and higher (> 10 nmol/L) levels of TCDD treatment (Fig. 1).

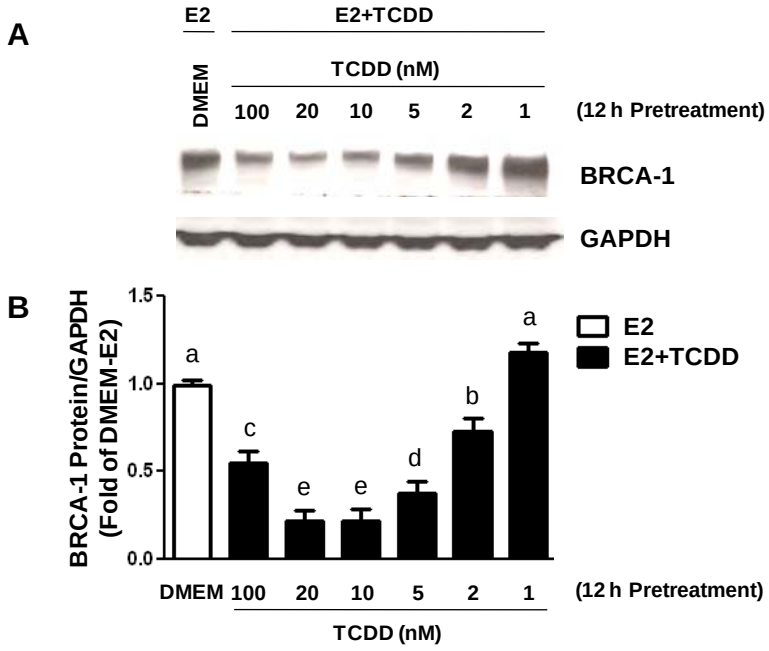


Fig. 1. TCDD reduces E2-dependent activation of BRCA-1 protein expression. (A) MCF-7 cells were precultured in DMEM or DMEM with various doses of TCDD for 12h. Then, cells were cultured for an additional 24 h in the presence of E2 (10 nmol/L) or E2 plus the corresponding TCDD concentration used in the pretreatment. Bands are immunocomplexes for BRCA-1 and GAPDH. In (B), bars are means ± SEM, n = 3 (CV < 5%). Means without a common letter differ, $P < 0.05$.

Based on these results, we selected the dose of 10 nmol/L TCDD for subsequent experiments. This concentration has been used previously to investigate the mechanisms of TCDD-induced initiation in breast epithelial cells, and the preventative effects of resveratrol [6]. We found that the pretreatment for 12 h with resveratrol followed by the cotreatment with resveratrol plus E2 for 24 h dose-dependently (10>1>0.5 μmol/L) prevented the repressive effects of TCDD on BRCA-1 protein (Fig. 2). The inhibition of BRCA-1 expression by TCDD (~40%) was completely reversed by the cotreatment with 1 μmol/L resveratrol. The latter dose is lower than the maximal levels (2.4 μmol/L) of resveratrol reported in pharmacokinetic studies with human subjects [7]. In keeping with earlier reports documenting estrogen-like effects of resveratrol at higher concentrations [8] (Fig. 2), a ~1.0-fold induction of BRCA-1 protein was observed in the presence of 10 μmol/L resveratrol plus E2 compared to treatment with E2 plus TCDD. To date, most of the in vitro experiments that investigated the effects of resveratrol in breast epithelial cells have utilized doses exceeding ~10-20 μmol/L [9].

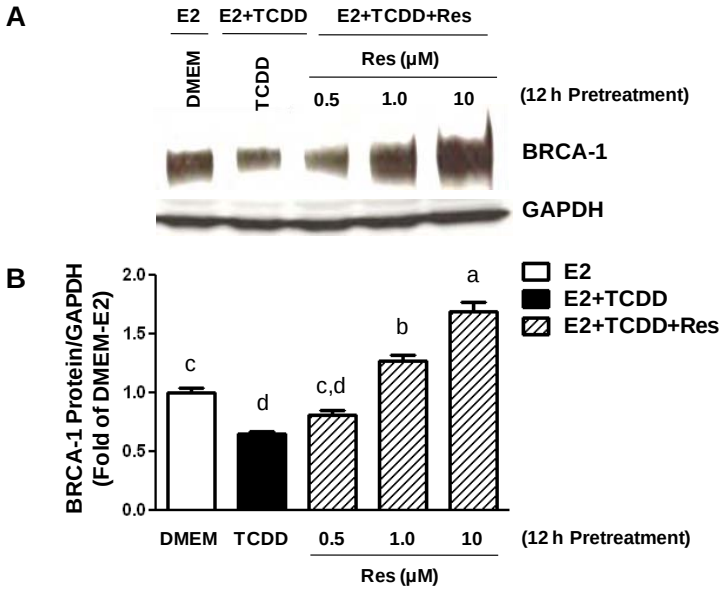


Fig. 2. Resveratrol antagonizes TCDD-dependent repression of BRCA-1 protein expression. (A) Breast cancer MCF-7 cells were precultured in DMEM, DMEM with 10 nmol/L TCDD, or DMEM with 0.5, 1, or 10 μmol/L resveratrol (Res) for 12h. Then, cells were cultured for an additional 24 h in the presence of E2 (10 nmol/L), E2 plus 10 nmol/L TCDD, or E2 plus 10 nmol/L TCDD plus the corresponding Res concentration used in the pretreatment. Bands are immunocomplexes for BRCA-1 and GAPDH. In (B), bars are means ± SEM, n = 3 (CV < 5%). Means without a common letter differ, $P < 0.05$.

Results of dose-dependent experiments depicted in Fig. 1 and Fig. 2 suggest the stoichiometric ratios among AhR ligands and relative binding affinity for the AhR (Res<TCDD) likely impact the E2-dependent regulation of BRCA-1 expression. These data validated our methodological conditions for measurement of BRCA-1 expression in vivo. In parallel experiments for objective b) we tested the influence of TCDD and Res on BRCA-1 nucleosome reorganization. We performed PCR and chromatin immunoprecipitation assay experiments. We found that (Fig. 3) Res induced a significant recruitment of Brg-1 and Brm suggesting that this compound may exert protective effects by changing the nucleosome reorganization.

Fig. 4a

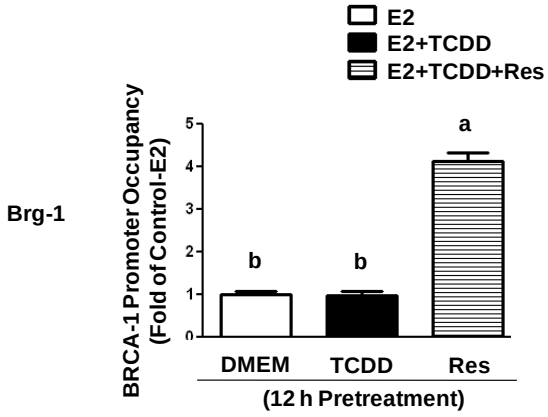


Fig. 4a

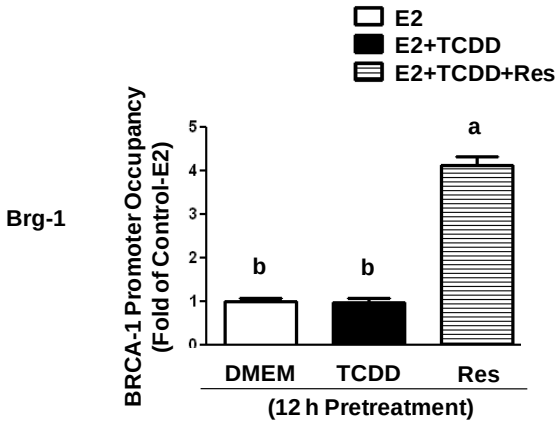


Fig. 3. Resveratrol induces the recruitment of nucleosome remodeling factors. RT-PCR and chromatin immunoprecipitation assays were performed using nuclear extracts obtained from MCF-7 cells pre-treated with TCDD or Res and then cotreated with TCDD or TCDD plus Res.

Fig. 4b

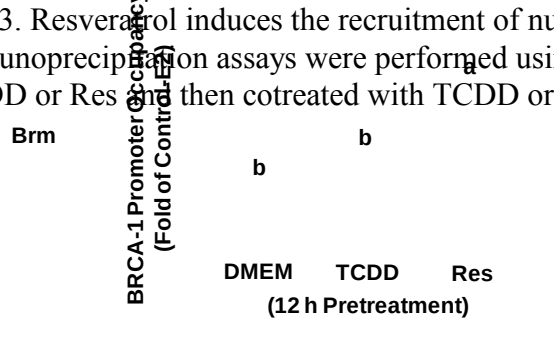
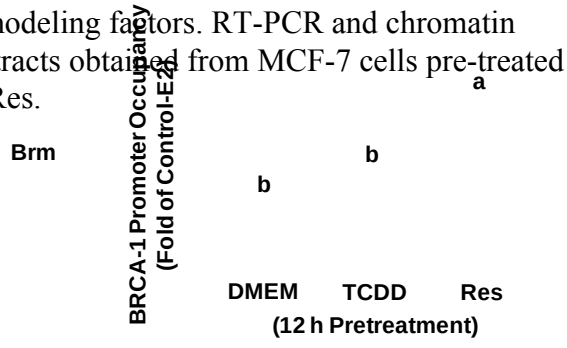


Fig. 4b



Objective c) of Task 1 deals with changes in the recruitment of histones and histones modifications including histone methylation. In control ChIP experiments, we observed that TCDD induced the recruitment of the AhR to the BRCA-1 promoter segment flanking XRE-2 (Fig. 4A, lane 2), whereas the intensity of the PCR amplification product was reduced following cotreatment with resveratrol (Fig. 4B, lane 3 and Fig. 4C). However, no AhR enrichment was observed following incubation of chromatin with IgG (Fig. 4B, lanes 3-6) irrespective of treatment, and no differences in input chromatin were recorded (Fig. 4B, lanes 7-9). Moreover, we performed DNA pull:down assay for the AhR using CYP1A1 oligonucleotides containing a consensus XRE (Fig. 4D). These data validated the experimental conditions for measurement of AhR activation and recruitment to XRE and ChIP experiments.

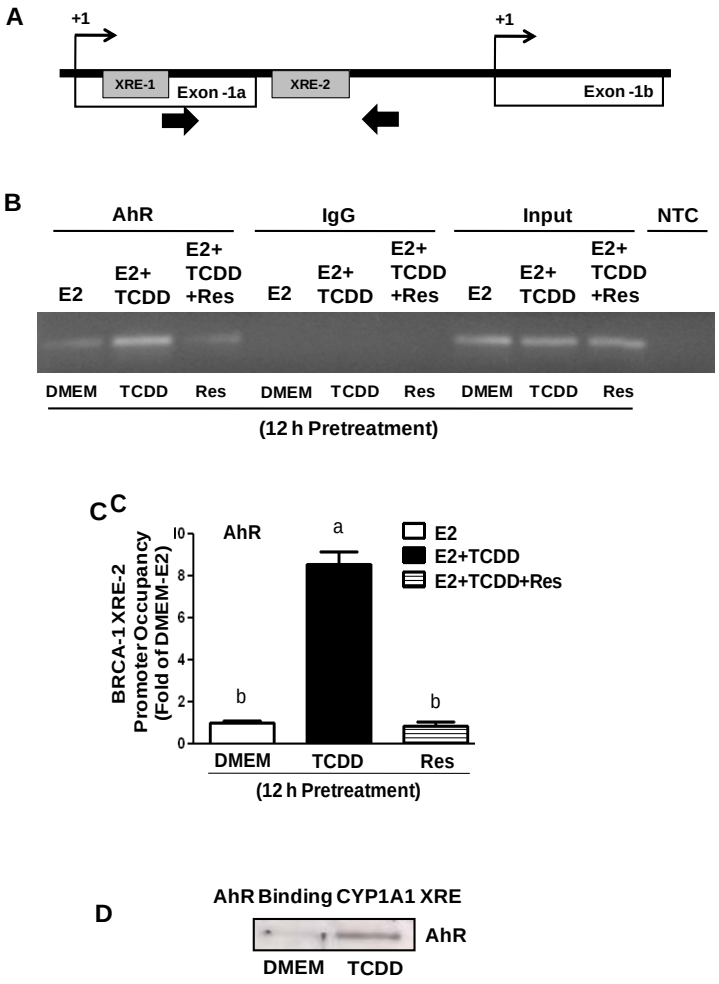


Fig. 4. Resveratrol antagonizes TCDD-dependent recruitment of the AhR to the BRCA-1 promoter spanning XRE-2. (A) Schematic representation of the BRCA-1 promoter. Arrows represent the position of oligonucleotides used to examine by ChIP assay the recruitment of AhR to the BRCA-1 promoter region flanking the XRE-2. (B and C) Pretreatment of MCF-7 cells with DMEM, 10 nmol/L TCDD, or 1 μmol/L resveratrol (Res) for 12 h were followed by cotreatment for 144 h with E2 (10 nmol/L), E2 plus 10 nmol/L TCDD, or E2 plus 10 nmol/L TCDD plus 1 μmol/L Res. (B) RT-PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. Inputs are control bands amplified from chromatin before immunoprecipitation. The size of the amplicon is 180 bp. (C) Bars are means ± SEM, n = 3. Means without a common letter differ, $P < 0.05$. (D) DNA protein-binding assay was performed by incubating nuclear extracts harvested from MCF-7 cells cultured in the DMEM or TCDD for 12 h and with CYP1A1 oligonucleotides.

Next, we examined how TCDD influenced the recruitment of factors to the BRCA-1 promoter segment comprising the start site of exon-1a (Fig. 5A). We found that TCDD stimulated the recruitment of the AhR (~8.0-fold) (Fig. 5B), MBD2 (~1.8-fold) (Fig. 5C), 3meH3K9 (~1.0-fold) (Fig. 5D) to this region. Also, TCDD induced the recruitment of DNMT1 (Fig. 5E), DNMT3a (1.8-fold) (Fig. 5F), and DNMT3b (0.9-fold) (Fig. 5G) to the BRCA-1 promoter. The addition of 1 μmol/L resveratrol in the pretreatment and cotreatment media reduced the recruitment of these factors to levels lower than those measured in DMEM-E2 chromatin samples.

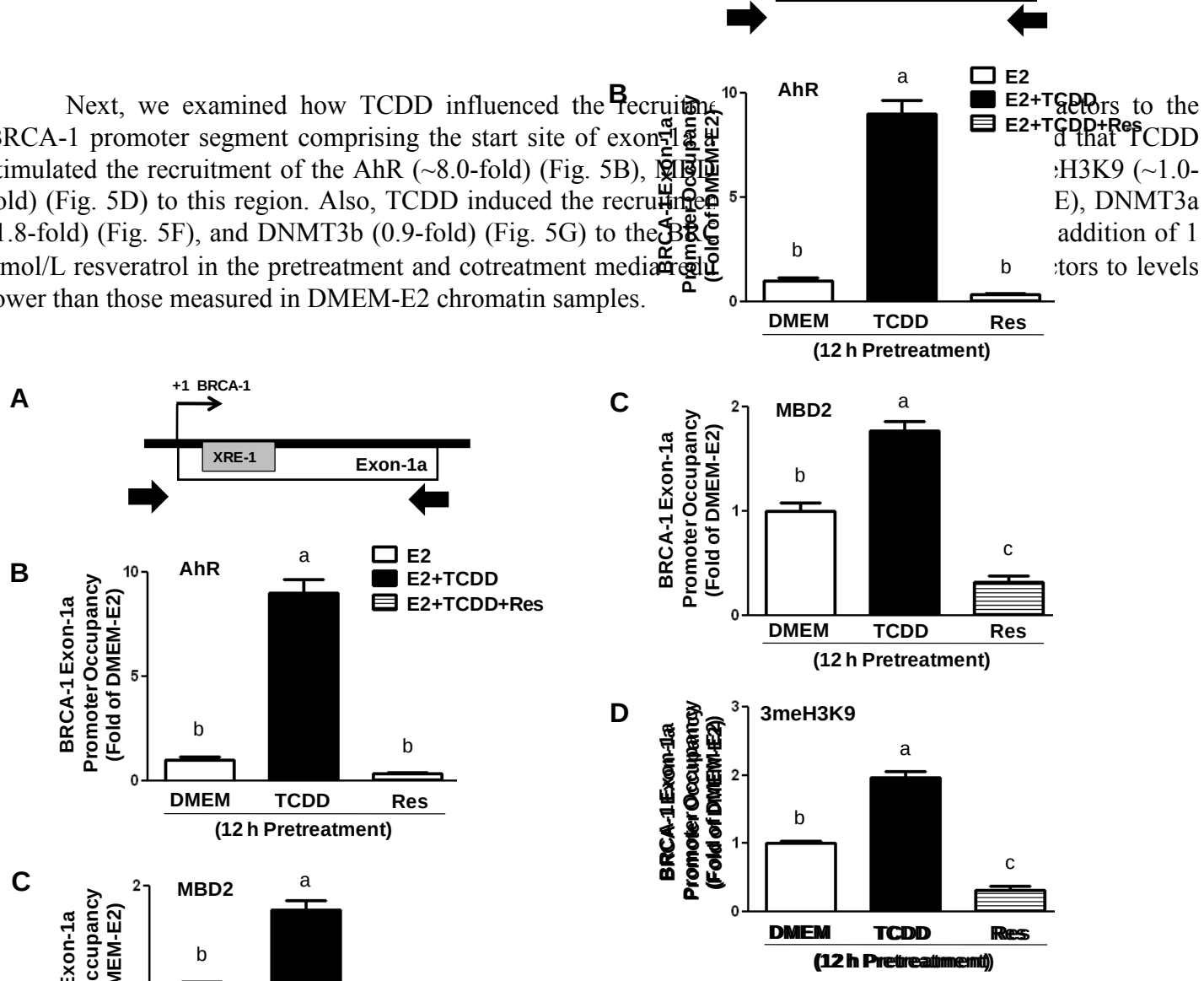


Fig. 5. Regulation of the (B) AhR, (C) MDB2, (D) 3meH3K9, (E) DNMT1, (F) DNMT3a, and (G) DNMT3b to the BRCA-1 promoter spanning exon-1a (A). Arrows represent the position of oligonucleotides used to examine by ChIP assay the recruitment of factors to the exon-1a region of the BRCA-1 promoter. Pretreatment of MCF-7 cells with DMEM, 10 nmol/L TCDD, or 1 μmol/L resveratrol (Res) for 12 h were followed by cotreatment for 144 h with E2 (10 nmol/L), E2 plus 10 nmol/L TCDD, or E2 plus 10 nmol/L Res. Bars are means ± SEM, n = 3. Means without a common letter differ, $P < 0.0$

As DNA methylation may impact adjacent chromatin domains [10], we examined the effect of TCDD treatment on recruitment of DNMTs to the BRCA-1 promoter segment comprising exon-1a (Fig. 6A). Results of ChIP analyses indicated TCDD enhanced (average ~1.0-fold) the recruitment of DNMT3a, DNMT3b (Fig. 6 B-D). Moreover, TCDD stimulated (~1.5-fold) the association of MBD2 and 3meH3K9 (Fig. 6E-F). Tri-methylated H3K9 is a suppressor chromatin marker coupled to DNA methylation by DNMT1 [11].

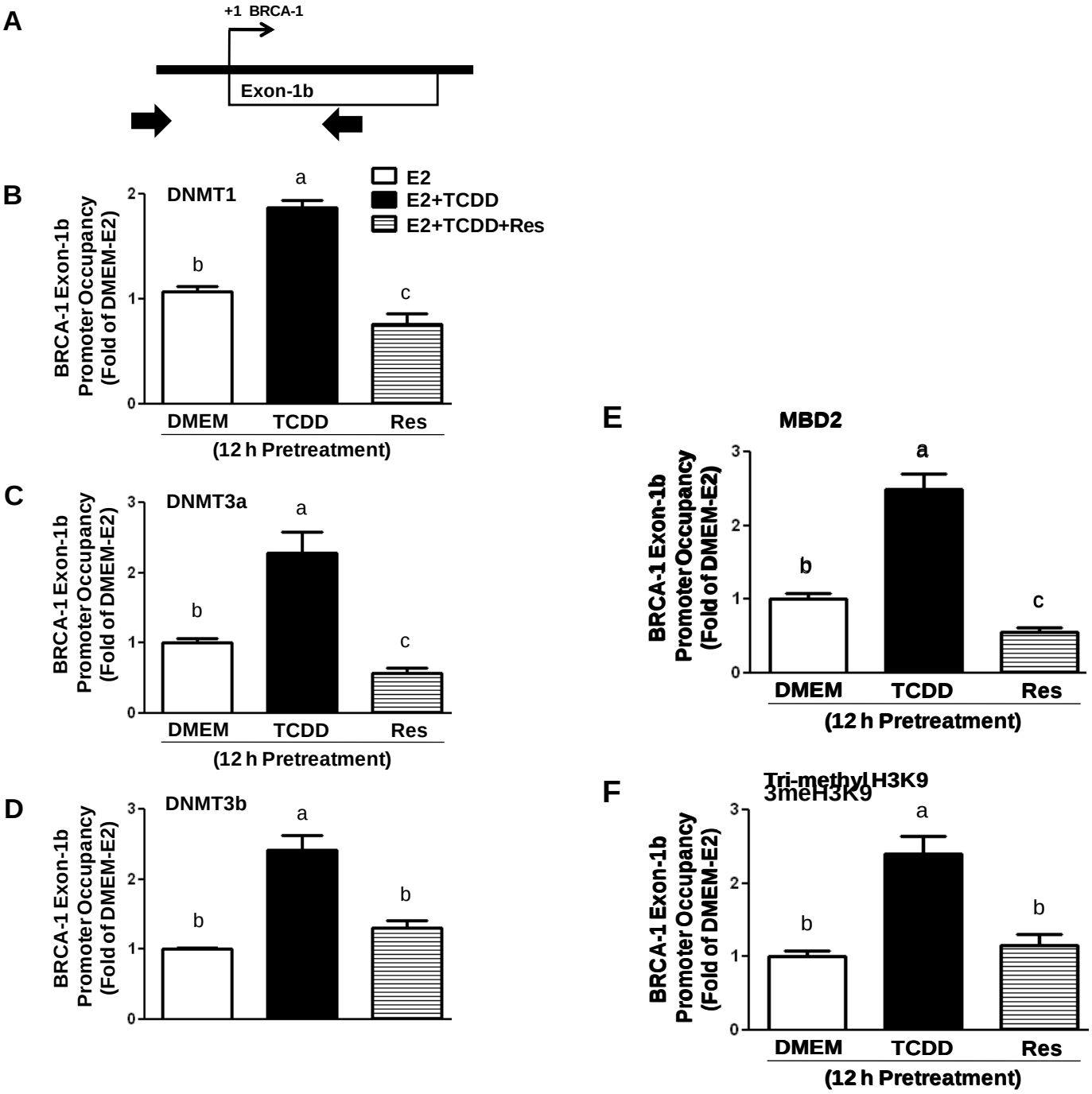


Fig. 6. Resveratrol prevents the TCDD-dependent recruitment of (B) DNMT1, (C) DNMT3a, (D) DNMT3b, (E) MBD2, and (F) 3meH3K9 to the BRCA-1 promoter spanning exon-1b (A). Arrows represent the position of oligonucleotides used to examine by ChIP assay the recruitment of factors to the exon-1b region of the BRCA-1 promoter. Pretreatment of MCF-7 cells with DMEM, 10 nmol/L TCDD, or 1 μmol/L resveratrol (Res) for 12 h were followed by cotreatment for 144 h with E2 (10 nmol/L), E2 plus 10 nmol/L TCDD, or E2 plus 10 nmol/L TCDD plus 1 μmol/L Res. Bars are means ± SEM, n = 3. Means without a common letter differ, $P < 0.05$.

Taken together, these findings suggested that recruitment of AhR to the BRCA-1 promoter segment flanking exon-1a was coupled to reduced BRCA-1 expression. The increased association of de novo (DNMT3a and DNMT3b) and maintenance (DNMT1) DNA methylation activities correlated with increased association of MBD2 and 3meH3K9. Conversely, resveratrol antagonized the TCDD-induced establishment of these epigenetic marks and reduction of BRCA-1 expression. Through these experiments, we validated the experimental methodologies necessary to test the effects of TCDD in vivo and gained important data about the role of preexposure to AhR ligands on repression of the BRCA-1 gene through epigenetic changes.

In utero treatment of pregnant rats with TCDD was performed 15 days post-conception. The treatment with resveratrol (RES) preceded TCDD treatment and continued until birth. Mammary, ovary, and liver tissue were collected at different time periods. The fourth abdominal mammary glands were dissected for whole mount preparations. Fig. 7 depicts the epithelial structures that were measured in whole mounts of mammary glands to assess the effects of treatments on mammary gland organization.

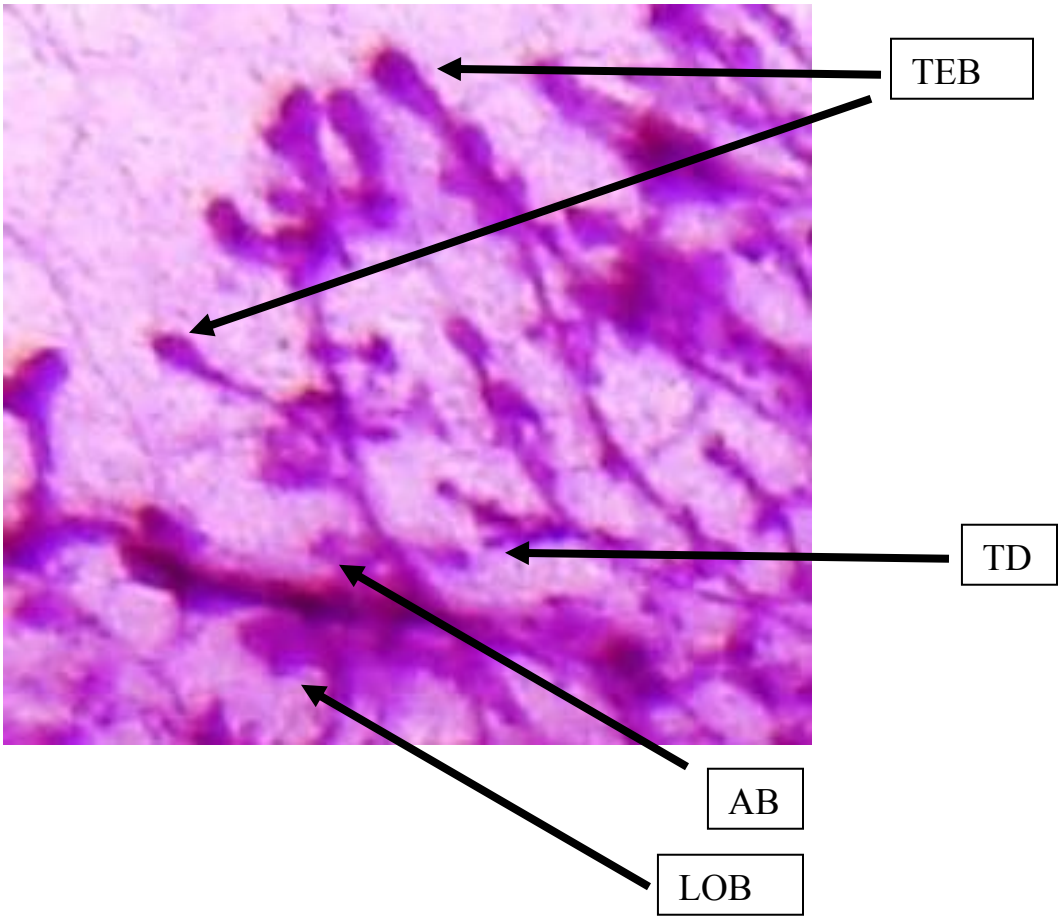


Fig. 7. Mammary gland whole mounts from 46PND female offspring. Images (20x magnification objective) were taken from the most exterior area opposite to the nipple and are representative of 5 animals per group. TEB: terminal end buds, TD: terminal ducts; AB: alveolar buds, representing differentiated epithelial structures; LOB, representing lobules.

Next, we assessed the total number of terminal end buds (TEB) and the relative density of lobulo-alveolar units using the procedure described in previous studies [12] (Fig. 8).

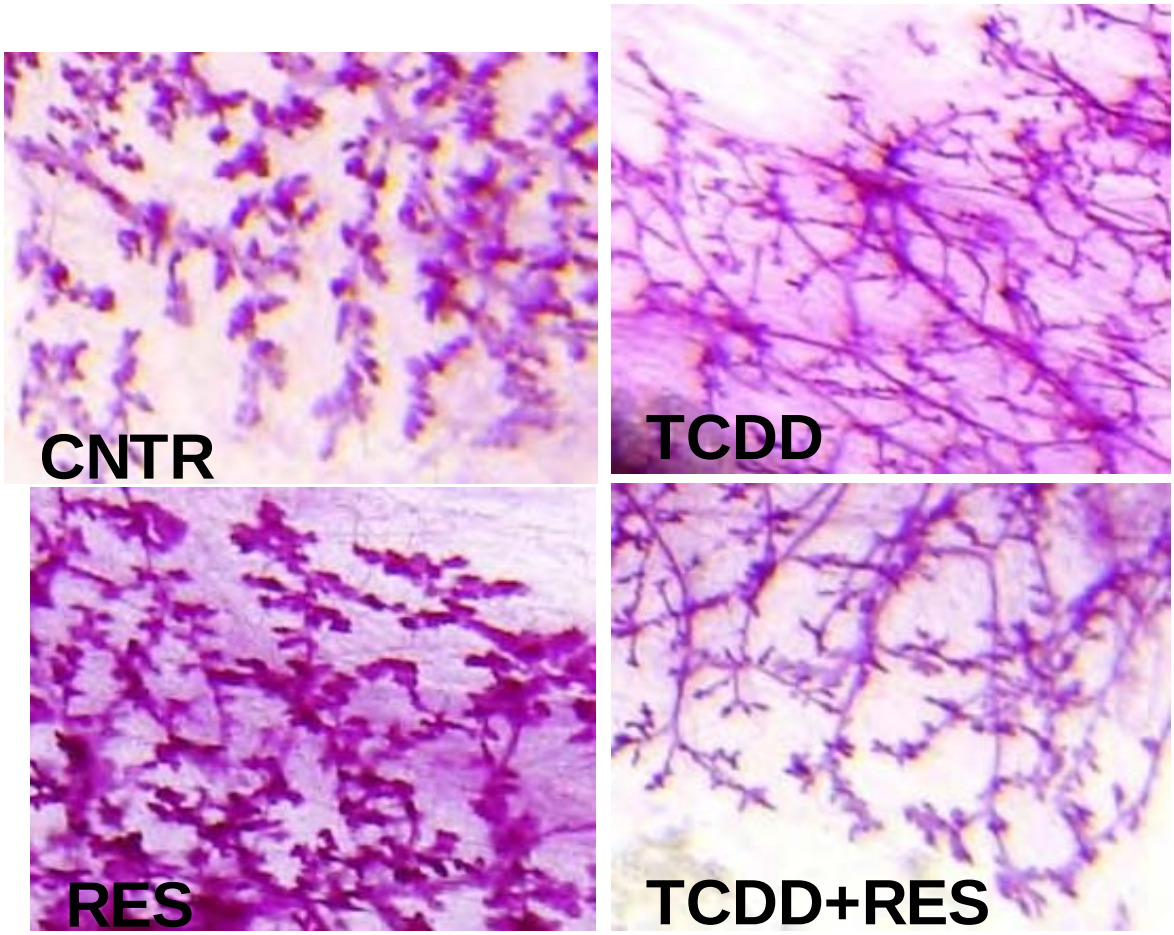


Fig. 8. Mammary gland whole mounts from 71 PND female offspring exposed *in utero* to 1) Control, 2) TCDD, 3) Res, and 4) TCDD+Res. Images were taken from the most external region opposite to the nipple, and are representative of 3-5 animals per group.

Terminal end buds (TEB), terminal ducts (TD), alveolar buds (AB) and lobules (LOB) were counted in the 5 mm most exterior zone opposite to the nipple. Between 3 and 5 individual glands were analyzed for each group. CNTR= control group receiving 0.2 ml sesame oil TCDD= animal received 1 µg/Kg BW TCDD on day 15 post conception by gavage; RES= Animal received resveratrol (100µg/rat/day) for 7 days before gavage with sesame oil, and then continued until end of pregnancy; TCDD+RES= animals were treated with both resveratrol and TCDD (Fig. 9). These results indicate that in utero exposure to TCDD induces changes in mammary gland organization. In particular, we found that TCDD induced the number of TEB compared to control, while reducing the number of LOB. These changes are indicative of a less differentiated mammary gland, which has been linked to increased risk of sporadic breast cancer [4]. Based on the success of this experimental methodology, we have collected mammary tissue at various periods of time and experiments are in progress to examine the time-dependent effects of TCDD exposure on mammary organization.

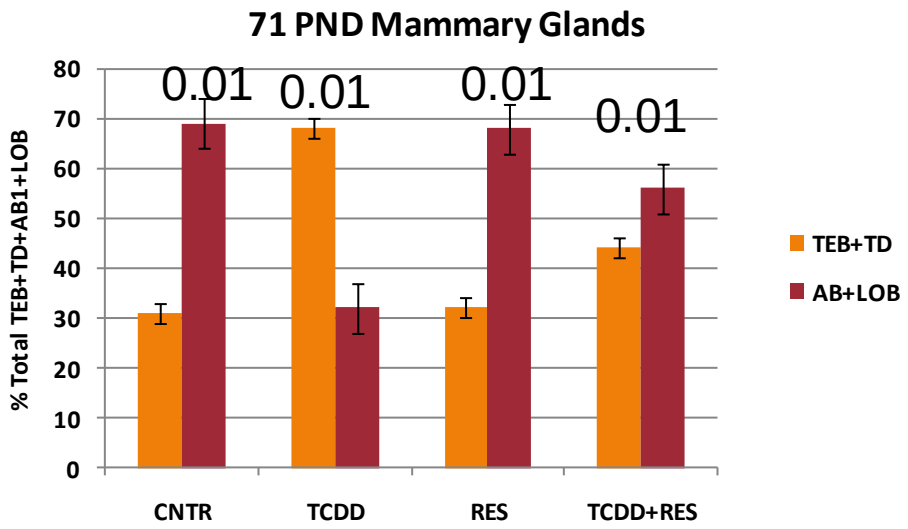


Fig. 9. In-utero exposure to TCDD changes mammary gland organization. Terminal end buds (TEB), terminal ducts (TD), alveolar buds (AB) and lobules (LOB) were counted in the 5 mm most exterior zone opposite to the nipple. Between 3 and 5 individual glands were analyzed for each group. CNTR= control group ($P<0.01$).

One of the key questions related to in utero exposure to TCDD is whether this influences the methylation status of the BRCA-1 promoter in female offspring. Therefore, we have optimized the technique and results are reported in Fig. 10. We cultured MCF-7 cells in the presence of various amounts of TCDD and resveratrol for 6 days. This time point has been used in previous studies to detect induction of CpG methylation by TCDD in the p16 promoter [13]. DNA samples obtained from MCF-7 cells were subjected to PCR analysis using methylated (M)- and unmethylated (U)-specific primers spanning the BRCA-1 transcription start site of exon-1a (Fig. 10A). This BRCA-1 promoter region harbors a CpG island reported to be methylated in sporadic breast tumors [14]. In the presence of E2, a band was amplified with unmethylated (U) primers, whereas the DNA amplification product of methylated (M) primers was undetectable (Fig. 10B, lane 2). The latter result was in agreement with the previously reported absence in MCF-7 cells of 5-methylcytosine in the BRCA-1 promoter region flanking exon-1a [15]. However, a PCR fragment was amplified with methylated-specific primers from DNA samples treated with 100 nmol/L TCDD (lane 4), but not in MCF-7 cells cotreated with TCDD plus 20 μmol/L resveratrol (Fig 10B, lane 6). Amplification with unmethylated and methylated-specific primers from control (Con) DNA corroborated the validity of the experimental conditions for methylation analysis and PCR amplification.

Based on these results, we tested the effects of lower levels of TCDD (10 nmol/L) and resveratrol (1.0 and 10 μmol/L). The results depicted in Fig. 10C suggested the treatment with 10 nmol/L TCDD induced hypermethylation of the BRCA-1 promoter (lane 4), whereas resveratrol exerted preventative effects (lane 6). The hypermethylation of the BRCA-1 promoter at 6 days correlated with reduced BRCA-1 protein expression in TCDD-treated cells, whereas the treatment with resveratrol reversed this repressive effect (Fig. 10D). Compared to treatment with E2 alone or E2 plus resveratrol, the addition of TCDD to the culture medium did not induce significant changes in the amplification of methylated fragments for the ERα promoter (Fig. 10E, lane 4).

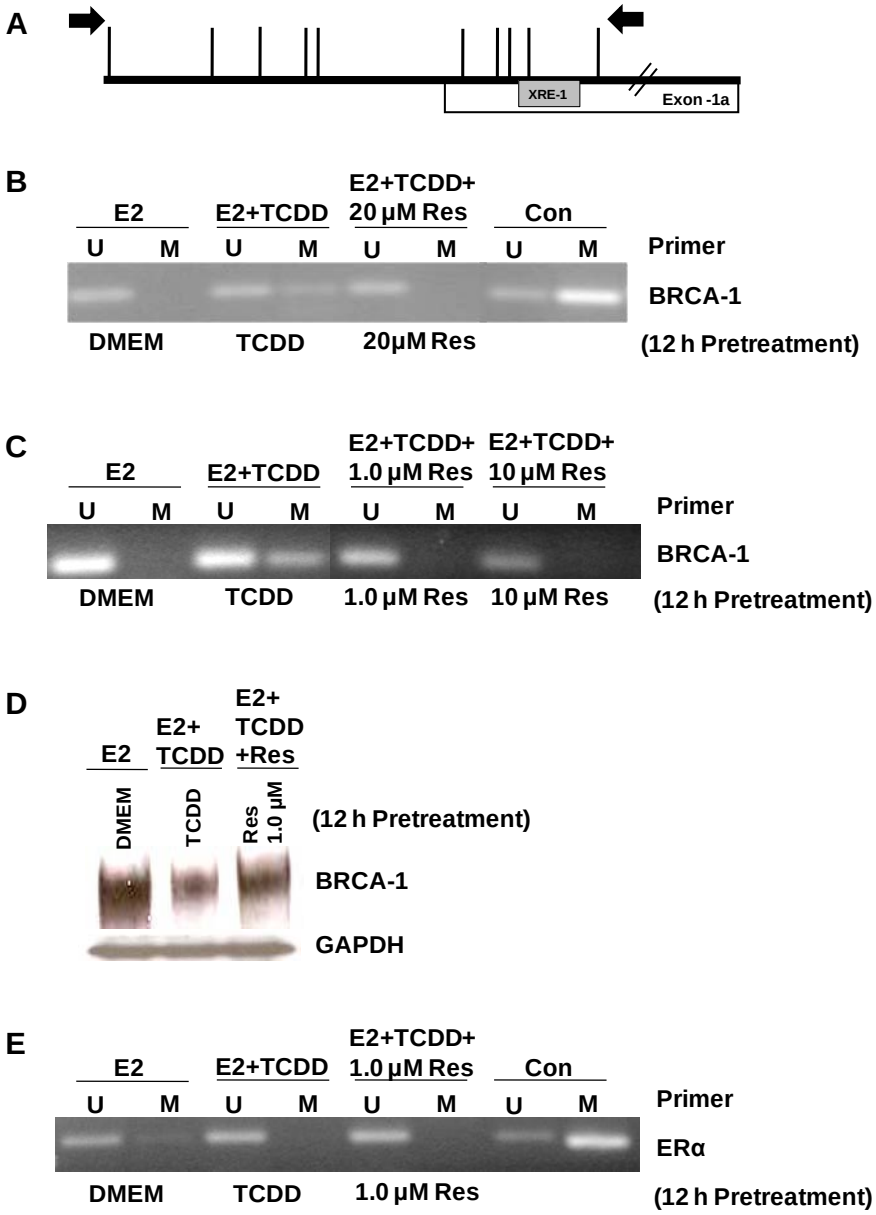


Fig. 10. Resveratrol prevents TCDD-induced methylation of BRCA-1 exon-1a. (A) Schematic representation of the BRCA-1 CpG island. Vertical bars indicate the location of CpG sites around exon-1a. Arrows comprise the BRCA-1 promoter region amplified by PCR. (B) MCF-7 cells were precultured in DMEM, DMEM with 100 nmol/L TCDD, or DMEM with 20 μmol/L resveratrol (Res) for 12h. Then, cells were cultured for an additional 144 h in the presence of E2 (10 nmol/L), E2 plus 100 nmol/L TCDD, or E2 plus 100 nmol/L TCDD plus 20 μmol/L Res. In (C) and (E), cells were treated as described in (B) except with doses of 10 nmol/L TCDD and 1 or 10 μmol/L Res. Genomic DNA was isolated and bisulfite-modified. DNA was analyzed for BCRA-1 (B and C) or ERα (E) promoter methylation using methylated specific PCR. U = unmethylated and M = methylated promoter sequences. Genomic DNA supplied by manufacturer was used as positive control (Con) for PCR. In (D), cells were pretreated with DMEM, 10 nmol/L TCDD, or 1 μmol/L resveratrol (Res) for 12 h followed by cotreatment for 144 h in the presence of E2 (10 nmol/L), E2 plus 10 nmol/L TCDD, or E2 plus 10 nmol/L TCDD plus 1 μmol/L Res. Bands are immunocomplexes for BRCA-1 and GAPDH.

The results of Fig. 10 are in agreement with those of other studies reporting the presence of unmethylated ERα promoter in MCF-7 cells [16] and pointed to promoter-specific mechanisms of regulation by the AhR. Experiments are in progress to examine the influence of in utero exposure to TCDD on BRCA-1 expression and promoter methylation status in mammary tissue of female offspring.

Key Research Accomplishments

1. Established experimental conditions documenting that TCDD reduces E2-dependent activation of BRCA-1 protein expression.
2. Obtained preliminary evidence resveratrol antagonizes TCDD-dependent repression of BRCA-1 protein expression and this is associated with induced recruitment of nucleosome remodeling factors.
3. Obtained preliminary evidence resveratrol antagonizes TCDD-dependent recruitment of the AhR, MDB2, 3meH3K9, DNMT1, DNMT3a, and DNMT3b to the BRCA-1 promoter. Reduced recruitment of these factors is indicative that AhR antagonists may be effective in preventing the loss of BRCA-1 expression seen in sporadic breast tumors.
4. Conducted in-utero exposure to TCDD and resvetarol and collected mammary tissue in female offspring. Obtained evidence TCDD reduces mammary gland structures associated with differentiation and increased terminal end bud structures associated with proliferation. Conversely, we observed a protective effect of resveratrol.
5. Developed BRCA-1 promoter methylation procedures to test DNA methylation of BRCA-1 in mammary tissue of female offspring.

Reportable Outcomes

1. Manuscripts:

   Andreas J. Papoutsis, Jamie L. Borg, Ornella I. Selmin, and Donato F. Romagnolo. BRCA-1 Promoter Hypermethylation and Silencing Induced by the Aromatic Hydrocarbon Receptor-ligand TCDD are Prevented by Resveratrol in MCF-7 Cells. In preparation, to be submitted to the Journal of Biochemistry.

2. Presentations:
   Donato F. Romagnolo. Loss of BRCA-1 expression: linking mechanisms of epigenetic silencing to nutrition and cancer prevention. Cancer Prevention Seminar Series, Arizona Cancer Center, March 9, 2010.

3. Degrees supported by this award:

   Dissertation project of Mr. Andreas J. Papoutsis, Ph.D. candidate in Nutritional Sciences and Cancer Biology, The University of Arizona, Tucson, AZ.

4. Funding applied based on work supported by this award:

   Dietary Influence on the Human Health Effects of Environmental Exposures (R21), RFA-ES-11-002. Grant Submitted : Dietary Modulation of Mammary Carcinogenesis Induced by Prepubertal Exposure to Dioxin. Co-Principal Investigators: Donato Romagnolo and Ornella Selmin.

5. Employment:
   Ornella Selmin, Research Associate Scientist.
   Jamie Borg: Research Technician

Conclusions

Taken together, preliminary findings suggest that recruitment of AhR to the BRCA-1 promoter segment flanking exon-1a leads to reduced BRCA-1 expression and BRCA-1 promoter hypermethylation. The increased association of de novo (DNMT3a and DNMT3b) and maintenance (DNMT1) DNA methylation activities correlate with increased association of MBD2 and 3meH3K9. Conversely, resveratrol antagonizes the TCDD-induced establishment of these epigenetic marks and reduction of BRCA-1 expression. In utero exposure to TCDD reduces the degree of mammary gland differentiation while increasing structures associated with increased proliferation. We have developed methodologies to examine BRCA-1 promoter methylation and experiments are in progress to continue measurements of changes of BRCA-1 expression induced by TCDD in mammary tissue of female offspring. No changes are requested on future work.

Evaluation of knowledge: the knowledge gained through the execution of the experiments reported here clearly suggest that exposure to AhR ligands is a risk factor in the onset of sporadic breast cancers through reduced BRCA-1 expression. Although preliminary, it appears that the mechanism responsible for AhR-dependent repression of BRCA-1 is promoter hypermethylation. This suggests that plausible medical products that may originate from this study are the need for preventing exposure to AhR ligands and the development of dietary strategies based on AhR antagonists. Task 2 of the project is evaluating whether loss of BRCA-1 expression induced by TCDD is linked to increased mammary cancer risk.

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Appendices

1. Front Page and Abstract of manuscript in submission to Journal of Nutritional Biochemistry

BRCA-1 Promoter Hypermethylation and Silencing Induced by the Aromatic Hydrocarbon Receptor-ligand TCDD are Prevented by Resveratrol in MCF-7 Cells

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Abstract

Epigenetic mechanisms may contribute to reduced expression of the tumor suppressor gene BRCA-1 in sporadic breast cancers. Through environmental exposure and diet, humans are exposed to xenobiotics and food compounds that bind the aromatic hydrocarbon receptor (AhR). AhR-ligands include the dioxin-like and tumor promoter 2,3,7,8 tetrachlorobenzo-p-dioxin (TCDD). The activated AhR regulates transcription through binding to xenobiotic response elements (XRE=GCGTG) and interactions with transcription cofactors. Previously, we reported on the presence of several XRE in the proximal BRCA-1 promoter, and that the expression of endogenous AhR was required for silencing of BRCA-1 expression by TCDD. Here, we document that in estrogen receptor-α (ER-α)-positive and BRCA-1 wild-type MCF-7 breast cancer cells, the treatment with TCDD attenuated 17β-estradiol (E2)-dependent stimulation of BRCA-1 protein and induced hypermethylation of a CpG island spanning the BRCA-1 transcriptional start site of exon-1a. Additionally, we found that TCDD enhanced the association of the AhR, DNA methyl transferases (DNMT)1, DNMT3a, and DNMT3b; methyl binding protein (MBD)2; and tri-methylated H3K9 (3meH3K9) with the BRCA-1 promoter. Conversely, the phytoalexin resveratrol, selected as a prototype dietary AhR antagonist, antagonized at physiologically relevant doses (1 μmol/L) the TCDD-induced repression of BRCA-1 protein, BRCA-1 promoter methylation, and the recruitment of the AhR, MBD2, 3meH3K9, and DNMTs (1, 3a, and 3b). Taken together, these observations provide evidence for a mechanistic role for AhR-agonists in establishment of BRCA-1 promoter hypermethylation and the basis for the development of prevention strategies based on AhR antagonists.

2. Proposed model for transcriptional regulation of BRCA-1 promoter.

(A) The activation of BRCA-1 by E2 is associated with unmethylated BRCA-1 promoter (open circles), the assembly of a p300/ERα/SRC-1 heterocomplex at an AP-1 site, the association of AcH4 and AcH3K9, and the recruitment of Sp1 and Sp4 to the Sp-binding region with constitutive presence of (CRE)-binding protein (CREB) at the cAMP response site. (B) The activation of the AhR (i.e. TCDD) and its recruitment to XRE lead to hypermethylation (black circles) of the BRCA-1 promoter. This is accompanied by the recruitment of MBD2, 3meH3K9, HDAC, and DNMTs, which methylate CpG sites and lead to epigenetic silencing. The epigenetic repression of BRCA-1 may be prevented by AhR antagonist (i.e. resveratrol).

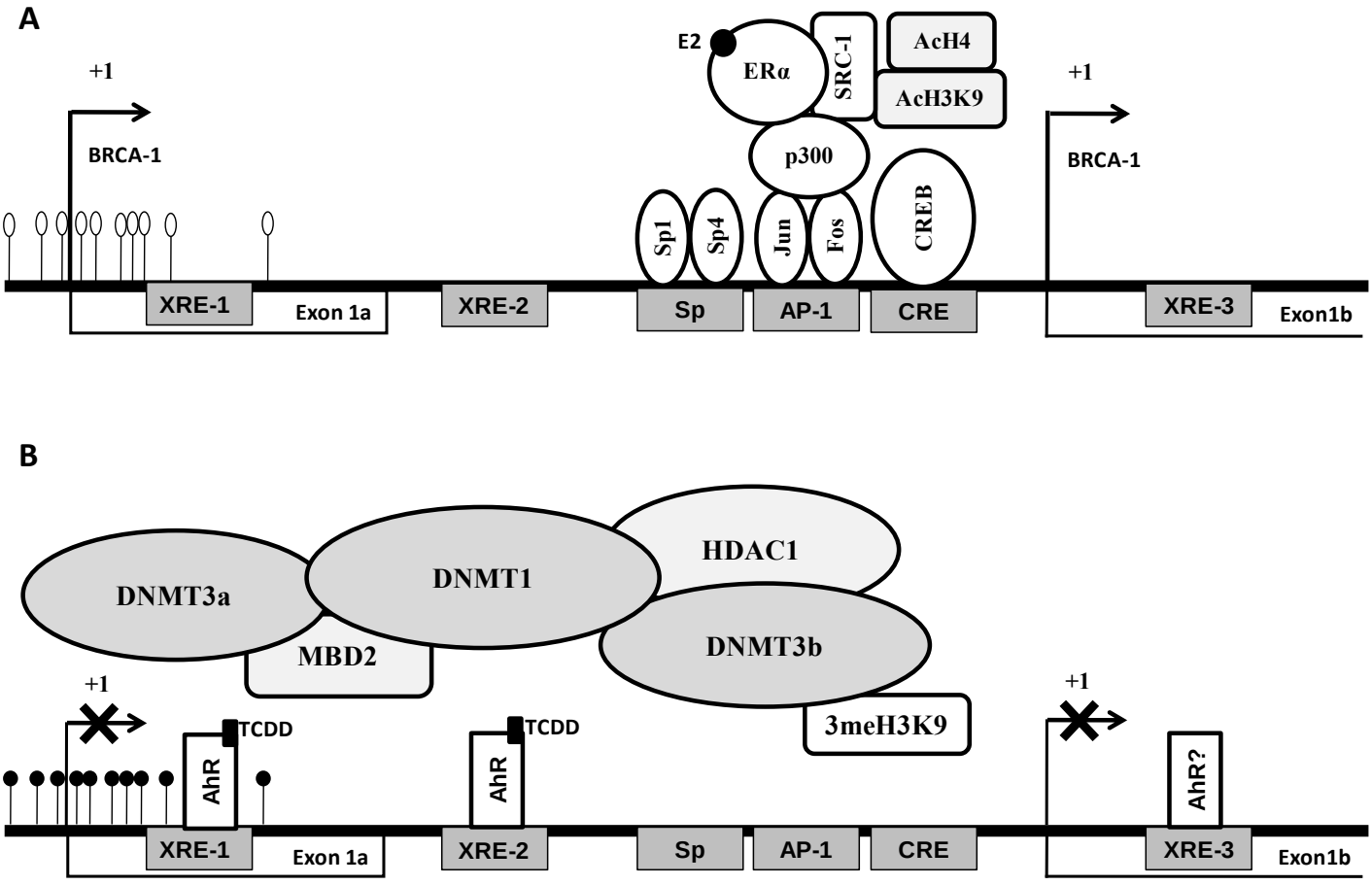

Fig 7 A and B